CAFFEINE EFFECTS ON FERTILIZATION AND DEVELOPMENT IN ARBACIA PUNCTULATA

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The effects of caffeine as a dietary factor upon the reproductivity and growth of mammalian species have been reported for rabbits by Stieve (1931), and for rats by Cheney (1944) and Bachmann et al. (1946), without uniformity in conclusions. None of these studies described the fundamental action of caffeine directly upon the gametes per se nor upon the early stages of development.

The effects of so many chemical factors in the environment upon the fertilization and development of the Arbacia egg are so well known, the comparative literature is so abundant, and the egg itself so ideal for studies of the effect of a substance upon permeabilities, cleavage rates, and developmental symmetry, that the Arbacia egg was chosen to investigate the critical factors underlying the influence of caffeine. The effects of caffeine were tested upon the gametes, zygote, and early developmental stages.

In 1945, it was reported by the author that caffeine, in concentrations of $M/1000$ (0.02 per cent) or above, depresses the O_2 consumption of the zygote as demonstrated by the Warburg-Barcroft micro-respirometer technique. It is the purpose of the current investigation to determine the relative sensitivity of the gametes to caffeine, its effect upon the cleavage stages and subsequent developmental phases through the mature pluteus larva, and the degree to which different concentrations retard and inhibit development.

METHODS AND MATERIALS

Arbacia punctulata gametes, shed directly into sea water (SW) and into eight different concentrations of caffeine-in-sea-water (CSW), after fifteen minutes were mixed for fertilization and allowed to develop to the following selected stages: fertilization membrane (FM), streak stage, 2-, 4-, 8-celled stage, late cleavage, blastula, gastrula, and pluteus larva. All eggs per experiment were obtained from the same female and all sperm from one male. Observations were made over a 72-hour period for the comparison of the developmental rate and form in SW and CSW. Normal time rates for the development of Arbacia were accepted as stated by E. B. Harvey (1940). The molarity series was $M/10$, $M/20$, $M/40$, $M/100$, $M/200$, $M/1000$, $M/5000$, and $M/10,000$ corresponding in concentration equivalents to 2.0, 1.0, 0.50, 0.20, 0.10, 0.02, 0.004, and 0.002 per cent of the alkaloid (caffeine Merck U.S.P., $C_8H_{10}O_2N_4 \cdot H_2O$, mol. wt. 212.21). This same procedure was followed by the author (1946-a) in obtaining preliminary data.

The experimental series to determine the relative sensitivity of the several stages (FM through pluteus) to a caffeine environment, was conducted in the following manner. Fertilized eggs were allowed to develop under normal conditions in SW

to the desired stages. Then several hundred specimens of each of the first eight stages cited above were transferred to each of the eight concentrations of CSW. The most advanced stage of differentiation attained by the experimentals in each concentration of CSW, and the rate and total time required to reach this degree of development, were compared with control eggs from the same female developed normally in SW throughout the 72 hours. Equivalent fluid volumes in stender dishes were maintained at the temperature of running SW. Conditions of significance, such as volumes, temperature, pH, and pressure, were constant for controls and experimentals in each series. Observations were made microscopically under a water immersion lens at 440 ×.

TABLE I

Effect of Caffeine on Fertilization and Development of Arbacia ova

Gametes shed into medium SW or CSW for 15 min. before mixing. N = normal in time and form of development to pluteus. Key: SW = normal sea water; CSW = caffeine in SW; FM = fertilization membrane.

%	Molarity	I Control N♀ XN♂ Dev. SW	II Exper. N♀ XC♂ SW	III Exper. C♀ XN♂ SW	IV Exper. C♀ XN♂ CSW	V Exper. C♀ XC♂ SW	VI Exper. C♀ XC♂ CSW
0.002	M/10,000	N	N	N	N	N	N
0.004	M/5000	N	N	N	N	N	N
0.02	M/1000	N	N	N	Slight retard. in early cleavage.	N	Many abnormal gastrulae. No plutei in 72 hours.
0.10	M/200	N	N	N or slight retard. 4-cell on but plutens normal form.	Retard. early cleavage. Arrested in late cleavage. No blastula. Cytolysis begun in 8 to 9 hours.	Normal blastula arrested in gast. Few early plutei —prism stage.	Ditto Col. IV with sharply localized echinochrome. Disintegration.
0.20	M/100	N	N	Time delay but plutei normal form.	Time retarded. Most div. abnormal. Arrested 4 and 8's when controls in 64 or late cleavage. Echinochrome localized. Dead.	Essential form N. Gastrula pluteus transition retarded several hours.	Ditto Col. IV with abnormal form in the 2-celled stage. Cells unequal.
0.50	M/40	N	Normal plutei time and form but pigment conc. noted and move slow.	Normal blast. Gastr. delay and arrest. Some cytolysis. No larva.	No FM or 1% FM. Egg oval. Pigment conc. begins in 2 to 3 hrs. and localized in 10 hrs. Then cytolysis.	Arrested in 16-cell to blast. Few abnormal gastrulae. No plutei before cytolysis.	Ditto Col. IV but develop pigment conc. in 5 to 7 hrs. Cytolysis.
1.0	M/20	N	Normal blast. but few reach gastr. No plutei.	Similar to Col. II but many abnormal cleavage and some cytol. in 6–7 hrs.	No FM. Eggs shape abnormal. Pigment clump in all within 1 hour. Cytolysis.	Early cleavage time retarded. Never beyond 16. Mostly less and abnormal.	Ditto Col. IV.
2.0	M/10	N	Time retard. 10% dev. into blast.	Time retarded. 16-cell maximum. Form abnormal. Arrested mostly in 4-celled. Pigment conc. Cytolysis.	No FM and ditto Col. IV for 1% given above.	Ditto Col. III but many arrested earlier in 2-cell. Maximum 8-celled rare.	Ditto Col. IV. No FM.

Results

Immersion of either or both the egg and sperm in any concentration of caffeine for 15 minutes prior to mixing the gametes did not render the egg non-fertilizable nor destroy the ability of the sperm to fertilize. These gametes, however, were not unaffected, at least by the higher concentrations, since with pretreated gametes (i.e. caffeinized eggs × caffeinized sperm), although they formed the FM when mixed and allowed to develop in SW, produced a zygote which never survived be-

Table II

Sensitivity to Caffeine Concentration

Dev. stage when transferred to caffeine	Term used is maximum development before death					
	0.02%	0.10%	0.2%	0.5%	1.0%	2.0%
I. Fertilization membrane	Gastrula to pluteus. Time retarded but form normal.	Blastula majority. Some gastrulae.	8 cells.	FM. No further dev.	FM. No dev.	FM. No dev.
II. Streak	Gastrula to pluteus. Time retarded but form normal.	Blastula majority. Some gastrulae.	16 cells.	No further dev.	No dev.	No dev.
III. 2-cells	Gastrula to pluteus. Time retarded but form normal.	32-celled and a few blastulae.	8 and 16.	No further dev.	No dev.	No dev.
IV. 4-cells	Gastrula to pluteus. Time retarded but form normal.	Blastulae fragmented badly.	16 and a few 32.	No further dev.	No dev.	No dev.
V. 8-cells	Gastrula to pluteus. Time retarded but form normal.	Blastulae less fragmented and a few gast.	16 and a few 32.	No further dev.	No dev.	No dev.
VI. Late cleavage	Gastrula to pluteus. Time retarded but form normal.	Blastulae.	Blast. move slow.	No further dev.	No dev.	No dev.
VII. Blastula	Gastrula to pluteus. Time retarded but form normal.	Gastrulae and "prism" triangles. No plutei in 3 days.	Gastr.	Abnorm. gastr.	Abnorm. gastr. Frag. badly.	No dev. Died in 3 hrs.
VIII. Gastrula	Gastrula to pluteus. Time retarded but form normal.	Triangular "prisms" only. No plutei, not even short prongs.	No dev.	No dev.	No dev.	No dev.

yond the early cleavage stages. Table I reveals the fact that typical plutei developed in normal time and form in all six combinations listed under columns I–VI inclusive with regard to molarities of M/5000 and M/10,000. The final line in Table I shows that the gametes, when shed into M/10 CSW (which approaches the maximum solubility concentration of this alkaloid in SW), and allowed to develop in SW, did form the FM but the zygote was retarded in its development and failed to form plutei before death. When shed into M/10 CSW and mixed for development also in CSW, the FM did not form, or if it did, it was not separated from the egg so as to be visible. Table I presents the various conditions under which the "first" retardation effects of any specific kind were observable. This table also shows the extent to which each concentration of caffeine interfered with the symmetry of normal growth.

The ability of the organism to carry on its normal metabolism in the presence of this trimethylated purine molecule is demonstrated by Table II, the Sensitivity Table. Eight typical stages of Arbacia development were transferred to a graded series of eight different concentrations of CSW. Table II lists only six molarities since it had been found in preliminary experiments by the author(1946-b) that M/10,000 and M/5000 CSW caused no response regardless of the organism's developmental stage when placed in CSW. All stages from FM through the Gastrula continued development to normal plutei in M/1000 CSW but the time schedule was retarded. The 2- and 4-celled stages were delayed more than the subsequent cleavage stages. M/200 CSW inhibited plutei formation from all stages. The maximum effect due to this concentration was similarly on the 2-celled stage and very few individuals ultimately exceeded the 32-celled stage. Other stages reached blastulation in M/200 CSW. Gastrulae formed "prisms" but no plutei. Higher molarities, M/100, M/40, M/20, and M/10 CSW, were more retarding and the last three (M/40, M/20, M/10 CSW) typically inhibited *all* further development beyond the stage attained when subjected to caffeine.

Discussion

Data by the author (1945) on the effect of caffeine upon the oxygen consumption of the fertilized Arbacia ova, recorded evidence that caffeine does affect the normal metabolism of the zygote. The effect of the molarities less than M/200 CSW in depressing the O_2 uptake of fertilized Arbacia eggs was slight and variable within 10 per cent, so they were not considered significant. Repetition of these experiments has emphasized again that molarities of M/200 CSW or above clearly depress the O_2 uptake as shown by Figure 1, the O_2 consumption—time relationship graph. These same molarities are the concentrations which are unquestionably effective in affecting the fertilization and the subsequent developmental time factor in the current study. In complete agreement are the effects of increasing concentrations of caffeine in depressing the O_2 uptake and in retarding the developmental time. The inhibition of the O_2 consumption effect of caffeine is undoubtedly a primary factor in the retardation of metabolism and cell division demonstrated by Table I. The possible mechanism by which caffeine interefers with the cellular metabolism, possibly by affecting only a single site in the pathway of the cytochrome oxidase-cytochrome chain of reactions, has been discussed previously by the author (1945).

There is some evidence that the egg is more sensitive than the sperm to caffeine. It will be noted by an examination of Table I that the lowest concentration, M/1000, at which any effect can be observed is under conditions where the caffeine-pretreated egg was fertilized by a non-treated sperm and developed in a CSW environment. The first effect with M/200 CSW involves the C♀ in M/200 CSW × N♂ and development in SW. The reverse, N♀ × C♂ M/200 CSW, with development in SW caused no retardation effect.
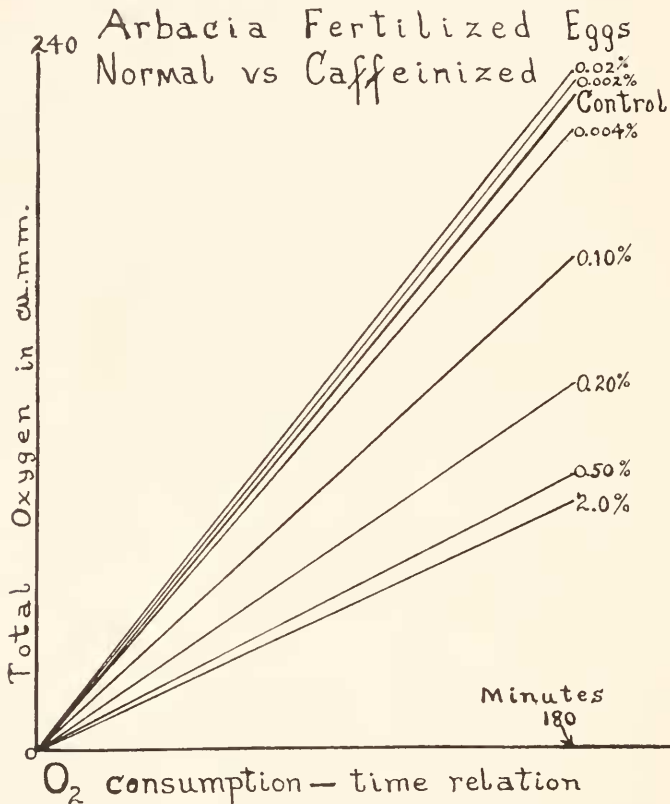

Figure 1

An osmotic effect of caffeine might be a cause underlying the sensitivity effects observed. Consequently, the eggs of a single female were subjected to the concentration series of the eight different molarities. Temperature was uniform by keeping the stender dishes in running sea water. The pH was not a factor since the differences between the normal SW, van't Hoff's artificial SW, and caffeine in either, did not increase the alkalinity more than 0.06 of a pH unit even in the highest concentration employed. This is well within the range, $6.0 - 8.3$, within which the Arbacia egg is fertilizable and normal development occurs as shown by Smith and Clowes (1924). Since the unfertilized Arbacia egg is a sphere, egg diameters were measured directly by an ocular micrometer and volumes were calculated by the

formula, $r^3 \times 4/3\pi$. In the case of the unfertilized egg alone, a total of over 5000 ova in the eight experimental media were examined. Detailed results employing the most drastic concentration (2 per cent) are cited in Table III.

Obviously, no significant effect can be concluded from the facts presented in Table III for the unfertilized egg. Similar statistics indicated also negative results with reference to the fertilized egg. A slight osmotic effect with time, if due to caffeine, could conceivably alter the transport of water which could modify the internal metabolism of the egg.

TABLE III

Diameter and volume variation in caffeinized (2% $\backsim$ M/10) Arbacia eggs

Egg No.*	30 *Minutes* after shedding eggs into				2 *Hours* after shedding eggs into				7 *Hours* after shedding eggs into			
	Sea water		2% Caffeine-in-SW		Sea water		2% Caffeine-in-SW		Sea water		2% Caffeine-in-SW	
	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000	Diameter in micra	Volume in cu. micra ×1000
1	71.0	187	71.0	187	71.0	187	72.6	201	71.0	187	72.6	201
2	72.6	201	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201
3	71.0	187	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201
4	72.6	201	71.0	187	72.6	201	72.6	201	71.0	187	72.6	201
5	71.0	187	71.0	187	71.0	187	72.6	201	71.0	187	74.3	214
6	69.3	173	71.0	187	72.6	201	71.0	187	72.6	201	74.3	214
7	71.0	187	72.6	201	72.6	201	71.0	187	72.6	201	71.0	187
8	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187	72.6	201
9	71.0	187	71.0	187	71.0	187	72.6	201	72.6	201	71.0	187
10	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187	71.0	187
Aver.	71.2	188	71.2	188	71.5	191	71.6	193	71.8	194	72.5	199

30 Min. normal vs. caffeinized (2%) Diameter difference is zero Volume difference is zero	2 Hr. normal vs. caffeinized (2%) Diameter difference is 0.1 mu Volume difference is 1400 = 0.73% increase	7 Hr. normal vs. caffeinized (2%) Diameter difference is 0.7 mu Volume difference is 5300 = 2.75% increase

No significant variation in 30 minutes, two hours, and seven hours.

* Each egg diameter listed is the average of 10 micrometer unit measurements on eggs from a single female. Final average of the 10 diameters cited in each column is actually the average of 100 eggs from the same female.

Advantage was taken of the procedures contributed to the literature by Harvey (1910), Lillie (1916, 1917, 1918), McCutcheon and Lucké (1926), Stewart (1931), and Jacobs and Stewart (1932), all of whom studied permeabilities employing the Arbacia egg as test material. Since rapid dehydration by strongly hypertonic solutions has destructive action on fertilized eggs, hypotonic solutions were used from which temporary immersion (30 minutes) results in complete recovery if transferred to normal SW.

Eggs from the same female were employed for both the unfertilized and fertilized series of experimental conditions. Several hundred ova, according to the series, were placed in stender dishes of normal SW (referred to here as NSW), van't Hoff's artificial sea water (ASW), bicarbonate—buffered to the pH of the NSW, hypotonic (40 per cent) sea water (40 ASW : 60 distilled water) indicated here as HSW, and HSW plus caffeine, i.e. CSW. The caffeine concentrations used were M/1000 (0.02 per cent), M 100 (0.20 per cent), and M/10 (2.0 percent). At the end of the first 30-minute period in any given experiment, approximately half of the eggs were transferred from CSW and divided approximately into equal masses to NSW and ASW for recovery studies. Readings (diameters) were made each minute for 10 minutes, followed by readings at 20, 30, and 60 minutes, and periodically for 24 hours although only the first 60 minutes were significant in the current study. Statistical tables similar to Table III were compiled. Molarities of M/1000, M/100, M/10 CSW in 40 per cent ASW were chosen because they represent significant concentrations as indicated by retardation effects of caffeine in Arbacia development—see Table I.

Since osmotic swelling is primarily one of diffusion, it is expected that when a cell is far from osmotic equilibrium with its environment, it will swell rapidly but the rate of swelling will decrease as equilibrium is approached. Volume changes were plotted against time. Thereby the velocity of swelling was noted. The rate of swelling was determined by the formula (McCutcheon and Lucké, 1926) in general use for this purpose; namely,

$$Kt = l_n \frac{V_{eq} - V_0}{V_{eq} - V_t}.$$

The experimental points were found to fall along a straight line as would be expected when

$$\log \frac{V_{eq} - V_0}{V_{eq} - V_t}$$

was plotted against time. The slope of the line gives the value of the velocity constant as k. This equation expresses the process of swelling in 40 per cent HSW and 40 per cent CSW for both unfertilized and fertilized eggs. This would be anticipated in a diffusion process.

The comparison of primary interest in the current paper is between data derived from the osmotic changes (rate and amount) specifically in HSW and the corresponding CSW. Comparison of these effects was also compared with the controls in NSW and ASW. No significant difference was evident in unfertilized eggs under the two hypotonic media of HSW and the corresponding CSW for any of the three concentrations of caffeine. Fertilized eggs show no difference under these experimental conditions. True, the fertilized eggs were more permeable than unfertilized eggs but the addition of caffeine had relatively no effect upon either series. *In other words, the HSW and the corresponding CSW results were equivalent.* Greater permeability to water and greater variability of fertilized eggs was expected since there is relative constancy of O_2 consumption before fertilization. The concentrations used have been shown earlier in this paper by the author to retard or inhibit cleavage completely. This fact indicates that the changes occurring in perme-

ability at the time of cleavage are of a different kind from those associated with cleavage furrow formation per se. The latter process is affected by caffeine which retards the O_2 uptake (Cheney, 1945).

The possibility of a viscosity effect of caffeine upon the Arbacia egg protoplasm, as a factor in explanation of the observed retardation of cleavage in caffeine solutions, has led to a study of the echinochrome stratification and egg deformation by centrifugation. The report upon these phenomena and the effect of caffeine upon the tension forces at the surface layers will be discussed in a later publication.

Summary

1. Caffeine does affect the gametes and the zygotes development of *Arbacia punctulata*.

2. Pre-treatment of both gametes with caffeine (0.5 per cent or higher) prior to mixing for fertilization and developed in CSW prevents the separation of the fertilization membrane.

3. Since some development occurs if both gametes are caffeinized but developed in SW, the FM may form but not be separated from the egg mass in the case of CSW as the environment.

4. The results of gamete activity following immersion and subsequent fertilization and development in CSW suggest that the egg is somewhat more sensitive than the sperm to caffeine.

5. Minimum effective concentrations in general are 0.1 per cent CSW (M/200). Slight retardation of development in 0.02 per cent CSW, under conditions of C♀ × N♂ and development in SW, is noted.

6. Oxygen uptake inhibition effect of caffeine is a primary factor in the retardation of cleavage time.

7. Early cleavage stages are retarded most.

8. The inhibition of O_2 utilization, retardation of cleavage time, and the degree of interference with the symmetrical differentiation attained, are directly proportional to the caffeine concentrations.

9. Concentrations of 0.5 per cent CSW (M/40) or higher as an environmental medium inhibit completely the physiological processes underlying the mechanics of further differentiation.

10. Caffeine does not initiate an osmotic effect with regard to the transport of water into the Arbacia egg.

11. Other factors in the explanation of the results observed are suggested.

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